CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Diuron

2.1.1 The use of diuron

Diuron, (3-(3,4-dichlorophenyl)-1,1-dimethylurea), is an herbicide belonging to the phenylamide family and the subclass of phenylurea. This substituted urea herbicide inhibits photosynthesis by preventing oxygen production and blocks the electron transfer at the level of photosystem II of photosynthetic microorganisms and plants. Diuron was introduced in 1954 by E.I. Du Pont de Nemours & Co. (Inc.) under the trademark "Karmex" (Liu, 2001). This compound has been used to control a wide variety of annual and perennial broadleaf and grassy weeds, as well as mosses. It has been also used on non-crop areas such as roads, garden paths and railway lines and on many agricultural crops such as fruit, cotton, sugar cane, alfalfa and wheat (Widehem *et al.*, 2002).

In Thailand, diuron is widely used in agricultural area. It was at the sixth rank of the imported hazardous chemical substances in 2003 (Table 2.1). Recent report, it was observed that diuron has been imported during January to August 2006 with the quality of 57,600 kg (13,622,933 baht). (Data from Customs Department, 2006)

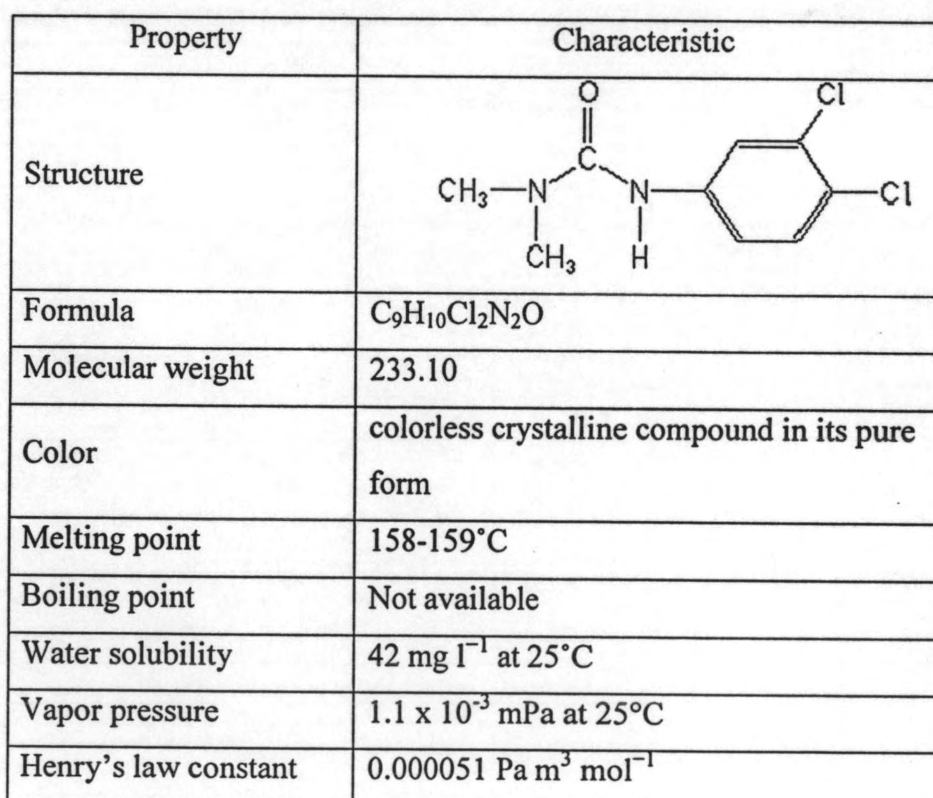
2.1.2 Physical and chemical properties

Physical and chemical properties of diuron were presented in the Table 2.2 (Giacomazzi and Cochet, 2004).

Table 2.1 Imported herbicides by value in 2003 (Department of Agriculture, 2003)

Rank	Herbicide	Quality (kg)	Value (Baht)
1	Glyphosate isopropylammonium	24,812,105	1,824,107,984
2	Paraquat	8,366,582	1,385,300,727
3	Ametryn	2,374,950	488,492,508
4	2,4-D	5,114,724	392,071,423
5	Atrazine	2,364,450	309,974,446
6	**Diuron**	**984,245**	**178,858,920**
7	Bromacil	304,309	173,592,721
8	Fenoxaprop-P-ethyl	210,658	150,816,860

Table 2.2 Physical and chemical properties of diuron

Property	Characteristic
Structure	
Formula	$C_9H_{10}Cl_2N_2O$
Molecular weight	233.10
Color	colorless crystalline compound in its pure form
Melting point	158-159°C
Boiling point	Not available
Water solubility	42 mg l^{-1} at 25°C
Vapor pressure	1.1 x 10^{-3} mPa at 25°C
Henry's law constant	0.000051 Pa m^3 mol^{-1}

2.1.3 Toxicity

Diuron is harmful to human and animals. Diuron is absorbed from the gastrointestinal and respiratory systems. The exposure to diuron can cause eye irritation, skin irritation and formation of methemogobin (methemogobin is an abnormal form of hemoglobin). Diuron has caused genetic damage in developing embryos and in bone marrow cells in mice. It also decreased the production of substances necessary for normal immune system function, and caused reduced birth weights when laboratory animals were exposed during pregnancy (Cox, 2003). Besides, the U.S. Environmental Protection Agency (USEPA) classifies diuron as a "known/likely" carcinogen because it has caused bladder cancer, kidney cancer and breast cancer in studies with laboratory animals (Cox, 2003; Giacomazzi and Cochet, 2004).

Diuron is slightly toxic to birds. In bobwhite quail, the LC_{50} is 1,730 ppm. In Japanese quail and ring-necked pheasant, the LC_{50} is greater than 500 ppm. On aquatic organisms LC_{50} (48 h) values for diuron range from 4.3 to 42 mg/l in fish and range from 1 to 2.5 mg/l in aquatic invertebrates. The LC_{50} (96 h) is 3.5 mg/l for rainbow trout. Thus, diuron is moderately toxic to fish and slightly toxic to aquatic invertebrates (Giacomazzi and Cochet, 2004).

2.1.4 Environmental fate

Due to its high persistence (one month to one year), diuron can be found in many environment such as soil, sediments and water. In soil, considering its low volatility and high K_{oc}, diuron is found in the solid phase rather than in the gaseous or liquid phase. Sorption studies of diuron have shown that the proportion of organic matter in soil directly influences the amount of adsorbed diuron (Giacomazzi

and Cochet, 2004). Diuron dissipation could also be leaching due to intensive rainfall, then leading to a main cause of groundwater pollution (Gooddy *et al.*, 2002). Diuron is also a source of pollution in aquatic environment because of its use as antifouling paint biocide. Diuron and diuron degradation products were detected in surface waters and in the bottom sediments, but few data were reported on the in situ biodegradation. In laboratory, diuron showed no biodegradation over 42 days in seawater at 15 °C, while the degradation products of diuron were less persistent (Giacomazzi and Cochet, 2004).

2.2 Physical and chemical treatment of diuron

There are several methods which are employed in diuron treatment. Physical and chemical treatment is a method which has been used for diuron removal.

Diuron has a very slow rate of natural hydrolysis in a neutral solution at 25 °C. However, degradation in water solution hydrolysis yields an irreversible reaction giving 3,4-dichloroaniline as the only product (Figure 2.1).

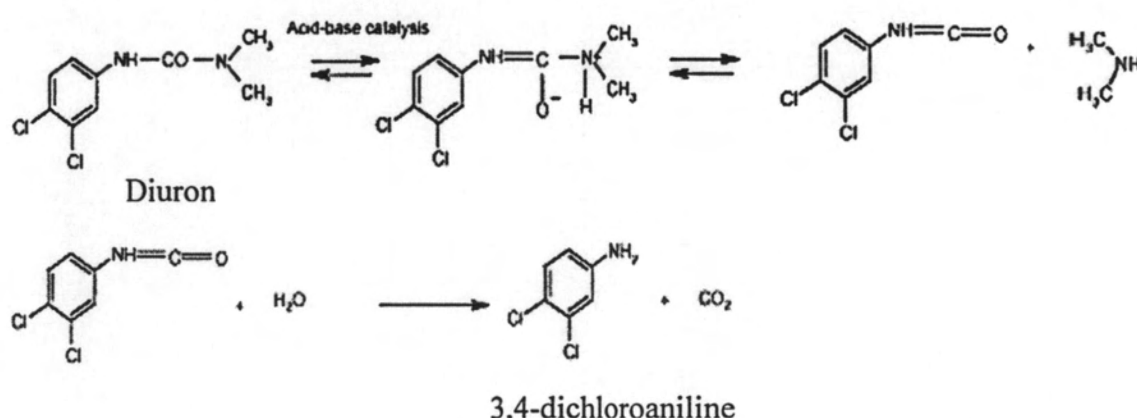


Figure 2.1 Predicted pathway reactions of chemical degradation of diuron (Salvestrini *et al.*, 2002).

Aqueous photolysis occurs through absorption of UV between 200 and 300 nm. Photodegradation of diuron by Photo-Fenton or TiO_2 system have been recently studied with the aim of reducing diuron concentration in water. Total mineralisation (i.e., complete disappearance of Total Organic Carbon (TOC)) can be achieved after a long irradiation (more than 200 min) (Malato *et al.*, 2002). However, it appears that 90% of the initial TOC could be mineralised in approximately 125 and 159 min, respectively, with each technique, providing an interesting way to explore further in wastewater treatment (Malato *et al.*, 2002). In contrast, the photodegradation of diuron under UV sunlight in natural seawater appeared very minor (Okamura, 2002).

However, one of the disadvantages of the physical and chemical treatments is their expensive cost. In addition, these treatments do not totally solve the contamination problem. The contaminants are incompletely treated. It transfers the contaminant form one phase to another phase which might become more toxic. Consequently, the secondary treatment is required for a complete treatment (Evans and Furlong, 2003).

2.3 Overview of bioremediation

Bioremediation is the complete degradation process and the most employed to change the molecular structure of organic compounds, thereby degrading toxic or potential toxic organic chemical into harmless compound. Moreover, this process can decrease or eliminate the diuron from the contaminated soil using microorganisms (King *et al.*, 1997). The advantages of bioremediation are often significantly less expensive when compared to other alternatives. In addition, it can reduce the time several fold compared to conventional technology as well as is a natural and mild condition (Alexander, 1994; Evans and Furlong, 2003).

2.3.1 Basic concept of bioremediation

1. Mineralization

Microorganisms are able to take impurity as nutrients for their metabolism and can metabolize them to carbon dioxide and water. Therefore, microbial activity treatment is necessarily needed to remove and demolish the impurity. In some case, the complete metabolization should be utilized by several microorganisms (Evans and Furlong, 2003).

2. Cometabolism

Microorganisms cannot utilize contaminant (co-substrate) as nutrient for their metabolism. Because of this, the specific enzyme is used for the target contaminant degradation. In some case, the enzyme reacted with another substrate (primary substrate) is necessary (Evans and Furlong, 2003).

3. Immobilization

In order to eliminate the contaminant, especially metal the adsorption or bioaccumulation of microorganisms or plants were widely used (Evans and Furlong, 2003).

Factors affecting bioremediation

The right microbe in the right place with the right environmental factors for degradation is necessarily needed for success in bioremediation methods. Bacteria and/or fungi, which have the physiological and metabolic capabilities, are widely used to degrade the contaminant (Boopathy, 2000). They especially summarized factors that affect the success of bioremediation as followed:

1. Energy source

One of factors that affect the success of the bioremediation is energy source. The activity of microorganisms playing the major role in the bioremediation processes is affected when the energy source is limited. In some case, the activity of microorganism is actively work by using pollutant as an energy source. Furthermore, more efficiencies of each degradation process depend on microorganisms (biomass, community diversity, enzyme activities for degradation of the pollutants), substrate (physical and chemical characteristics, molecular structure, and concentration) and environmental stresses (pH, temperature, moisture content, availability of electron acceptors and carbon and energy sources).

2. Bioavailability of the contaminant

The rate of biodegradation is the contaminant uptake and transfer rate dependent of microorganism. The bioavailability of pollutant is actively controlled by the physical and/or chemical processes, i.e. biosorption and desorption, diffusion, and dissolution. Decreasing of the bioavailability may result from: firstly, the chemical reactions between the contaminants and the natural organic substance. Secondly, rate of diffusion into very small pores and absorption into organic substance and, finally, the formation of semi-rigid films around non-aqueous-phase liquids (NAPL) with a high resistance toward NAPL-water mass transfer. Indeed, increasing the availability of contaminants for microbial degradation, surfactants can be used.

2.3.2 Natural attenuation

The simplest method of bioremediation to implement is natural attenuation, where contaminated sites are only monitored for contaminant concentration to assure regulations that natural processes of contaminant degradation

are active (Kaplan and Kitts, 2004). This strategy is advantageous as low cost (Alexander, 1994). Because of low population size of the indigenous degrading microorganisms, natural attenuation often takes a long time to completely degrade the pollutants (Forsyth *et al.*, 1995).

2.3.3 Biostimulation

Biostimulation treatment requires adjustments to the site (contaminate soil or water) in order to provide bacterial community with a favorable environment in which they can effectively degrade contaminant. The process is stimulated by addition of oxygen and nutrients, such as carbon, nitrogen and phosphorus and other biostimulating agents (Boopathy, 2000; Kaplan and Kitts, 2004).

2.3.4 Bioaugmentation

The fact that sometimes the indigenous microorganisms do not have ability to seed up the toxic degradation strategies therefore it is necessary to add the contaminant-degrading bacteria or specialized microorganisms as either a pure culture or a mixed culture for the treatment, called bioaugmentation (Al-Awadhi *et al.*, 1996; Van-Limbergen *et al.*, 1998). The advantage of this treatment is that the biodegradation can be occurred immediately; therefore the wipe up time is reduced (Richard and Vogel, 1999).

The major concerning conditions for bioaugmentation are include: firstly, low of indigenous bacteria that are able to degrade the target pollutants are less than 10^5 CFU per gram of soil (Providenti *et al.*, 1993). Secondly, the period time of decontamination is one of main factors. Therefore, adding the degrading bacteria could be used to start the remediation process with little or no lag period in order to shorten the determinant period. It is short time for decontamination (Molnaa and Grubbs, 1989). Thirdly, it provides a measure of assurance which the correct bacteria

were represented in sufficient number for the degradation (Molnaa and Grubbs, 1989). Finally, the physical or chemical treatment is needed to biodegrade the complex waste types before bioaugmentation (Forsyth *et al.*, 1995).

Moreover, Vogel (1996) demonstrated about the important parameters for bioaugmentation required the three main factors which are the bioavailability of the pollutant, the survival and activity of the added microorganism(s). For example;

a. Pollutant characteristic: bioavailability, concentration and microbial toxicity.
b. Soil properties: pH, moisture, organic matter and clay content.
c. Microbial ecology: presence of predators and competition.
d. Microbiology: the presence of co-substrates and enzyme stability and activity.
e. Methodology: inoculation concentration and method of inoculation.

2.4 Diuron biodegradation and bioremediation

The microbial degradation of the substituted phenylureas usually leads to the corresponding aniline resulting from the aliphatic chain hydrolysis. Dalton et al. (1966) have proposed an aerobic degradation pathway for diuron involving two successive *N*-demethylation reactions followed by the cleavage of the amide bond.

Cullington and Walker (1999) studied on the soil that degraded diuron rapidly after receiving three consecutive diuron retreatments, at a concentration of 40 mg/kg. The first two incubations lasted for 6 days, and the third for 2 days. These successive diuron applications were degraded rapidly, with residues from the third retreatment reduced from 47.3 to 6.1 mg/kg in 2 days.

Widehem *et al.* (2002) reported that *Arthrobacter* sp. N2 isolated from soil by enrichment procedures was able to metabolize diuron in pure culture. Diuron completely disappeared after 30 hours as it was the unique nitrogen source, after 50 hours as it was the unique carbon source, and after 70 hours as it was the only carbon and nitrogen source.

Sorensen *et al.* (2003) wrote a mini-review on bacterial strains and mechanisms involved in phenylurea herbicides degradation. The metabolic pathway of diuron degradation is in Figure 2.2

Dellamatrice and Monteiro (2004) found that a consortium of three bacteria, *Acinetobacter johnsonii* and two *Bacillus spp.* was isolated in medium containing diuron as the only carbon source. Diuron degradation was low in the sample without diuron application, however in the sample with three-year diuron application there was an increase, about seven times, in the degradation of diuron.

On the other hand, bioremediation of diuron in soil is rarely reported. Widehem *et al.* (2002) studied bioremediation of diuron 0.8 g/kg soil. Diuron begins to decrease after 16 days and there is 65% of diuron left in the soil after 30 days.

Anaerobic conditions

diuron

aerobic conditions

3,4-dichloroaniline

anaerobic pathways

aerobic pathways

O_2, NH_3

and/or

3,4-dichloro-N-(3,4-dichlorophenyl)benzamide

dichlorobenzene

Dichlorocatechol

or

and/or

3,4-dichloroacetanilide

Chlorocatechol

COOH—CH_2—CHCl—COOH

COOH—CH_2—CH_2—COOH

Figure 2.2 Metabolic pathways of bacterial degradation of diuron and 3,4-dichloroaniline (Sorensen *et al.*,2003).

2.5 Total microbial activity study

Dehydrogenases are oxidoreductase enzymes that take part in respiration in microbial cells. These enzymes oxidize organic compounds by the transfer of electron pairs from a substrate to nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) forming NADH or NADPH, respectively (Smith and McFeters, 1997). This vital part of the electron transport system of a cell (Crane *et al.*, 1991). The measurement of microbial dehydrogenase activity in soil and sediments has been used extensively as dehydrogenases are intercellular to the microbial biomass, common throughout microbial species and are rapidly degraded following cell death (Somerville *et al.*, 1987).

The use for measuring redox reactions in the cells (Smith and McFeters, 1997). The 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) has been used as the substrate for the dehydrogenase activity. INT successfully competes with NAD^+ and $NADP^+$ for electrons. INT inserts between ubiquinone and cytochrome *b* in the electron transport chain (Maurines-Carboneill *et al.*, 1998). As INT accepts electrons, it is reduced to a 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium formazan (INF). Colorimetric methods ased on INT reduction provide an accurate assay of dehydrogenase activities under both anaerobic and aerobic conditions (Trevors, 1982; vonMersi and Schinner, 1991; Bhupathiraju *et al.*, 1999).

Mosher *et al.* (2003) reported that to examine the potential for bioremediation of industrially contaminated sediments in the lower Mahoning River of northeast Ohio, USA, microbial activity (aerobic and anaerobic) measurements were made by estimating dehydrogenase activity using 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride. The result, the modified method optimized INT reduction

on concentration, time-course and temperature. The study is compared microbial activity determined by INT reduction from different sited show wide range of reduction rates.

Klose *et al.* (2005) studied about the enzyme activities in a sandy loam soil after fumigation with methyl bromide or alternative biocides. They found that the activities of dehydrogenase were significantly affected by the fumigant treatment and the sampling time.

Kaimi *et al.* (2006) studied that the biodegradation rate of diesel oil contaminated soil by the phytoremediation. The result showed that there was significant difference between the measurements of residual total petroleum hydrocarbon (TPH) in planted and unplanted soil. For the study of microbial activity, the number of aerobic bacteria was enumerated by using the plate count technique. It showed that the number of aerobic bacteria in planted and unplanted soil were different. In addition, the soil dehydrogenase activity was also studied for the microbial activity study by INT reduction technique. The values of soil dehydrogenase activity in planted soil were significantly different from the soil dehydrogenase activity in unplanted soil. This indicates a significant correlation between the enhanced the decrease of diesel oil in the phytoremediation and the number of aerobic bacteria and amount of dehydrogenase activity.

2.6 Microbial community analysis

Microbial communities provide useful data for studying both applied and basic environmental events. Microorganisms are present in virtually all environments and are typically the first organisms to react to chemical and physical changes in the environment. However, there is a serious lack of information on the effect of urea

herbicides on soil microbial communities. In general, the effect of herbicides on soil microbial communities has often been studied by conventional methods based on cultivation of the microbial communities and on measurements of their metabolic activities (Wardle, 1990). Recent developments in molecular analysis of bacterial communities have offered new tools for the exploration of highly diverse intestinal ecosystem (Tannock, 2001).

This study used PCR single-strand conformation polymorphism (PCR-SSCP) of 16S rDNA fragment to determine soil bacterial communities. PCR-SSCP is one of the techniques most widely used to identify a mutant sequence or a polymorphism in a known gene. The SSCP is based on the principle that the denatured samples lose their double-stranded configuration, and their folded single-stranded secondary structure emerges. These partly folded structures are dependent on the primary sequence, and move at different rates through a non-denaturing gel. The original SSCP protocol uses the incorporation of radioactive label and polyacrylamide gel electrophoresis on sequencing gels for detection (Orita *et al.*, 1989), and is labour intensive and time-consuming. Simpler methods using polyacrylamide gel electrophoresis and non-radioactive staining have been proposed; however, to improve the resolving power of SSCP it was necessary to use a variety of methods, such as adding glycerol, reducing temperature, increasing the length of the gels or the duration of the gel runs (Sentinelli *et al.*, 2000).

Radianingtyas *et al.* (2003b) were used PCR-SSCP to detected bacterial community dynamics in the biofilm reactor degrading 4-chloroaniline. PCR based single strand conformational polymorphism of 16 s rDNA and traditional cultivation procedures indicated that the bacterial composition in the reactor shifted in response to applied hydraulic retention time.

Since, previous studies are focused on diuron degradation in liquid solutions more than in soil. The information of diuron contaminated soil and its degradation would open the prospect of diuron remediation and improve soil quality. In this study, we were interested in the bioremediation of diuron contaminated soil by 3 approached, namely natural attenuation, biostimulation and bioaugmentation. The types of soil and microbial communities that affected diuron degradation were also studied.